8EHQ-0999-1157

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Eastman Chemical Company
P. O. Box 431
Kingsport, Tennessee 37662

September 16, 1999

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CERTIFIED MAIL
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Ladies and Gentlemen:

Eastman Chemical Company submits the following reports as required under TSCA §8(e) for your consideration.

> Di-2-Ethylhexyl Phthalate - Two-Generation Reproduction Toxicity Range-Finding Study in Wistar Rats: Continuous Dietary Administration

The 8(e) reference number for this substance is **8EHQ-91-1157**. A preliminary report on this study was submitted by Eastman Chemical Company on July 10, 1998.

If you have questions, you may contact me by telephone at (423) 229-4274 or the technical contact, Karen R. Miller, Ph.D., at (423) 229-1654.

Very truly yours,

F. David Petke, Ph.D.
Senior Technical Associate
Product Safety and Stewardship

cc: 8(e) file

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STUDY TITLE

Report

Di-2-ethylhexyl Phthalate - Two-Generation Reproduction Toxicity Range-Finding Study in Wistar Rats
Continuous Dietary Administration

DATA REQUIREMENT

87/302/EEC
OECD Guidelines, No. 416
OPPTS 870.3800

AUTHORS

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Dr. K. Deckardt
Dr. Chr. Gembardt
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STUDY COMPLETED ON

June 17th, 1999

PERFORMING LABORATORY

Department of Toxicology of
BASF Aktiengesellschaft
D-67056 Ludwigshafen, FRG

LABORATORY PROJECT IDENTIFICATION

15R0491/97096

VOLUME I OF III
(REPORT SECTION AND SUMMARY TABLES)

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GLP STATEMENT

Title: Report: **Di-2-ethylhexyl Phthalate** - Two-Generation Reproduction Toxicity Range-Finding Study in Wistar Rats; Continuous Dietary Administration

This study was conducted in accordance with the GLP provisions of the "Chemicals Act" (Chemikaliengesetz; Bundesgesetzblatt 1994, Teil I, 29.07.94; FR Germany) and the OECD Principles of Good Laboratory Practice (Paris, 1981).

June 17, 99

(Study Director)

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From the Department of Toxicology of
BASF Aktiengesellschaft, Ludwigshafen/Rhein, FRG
Head: Prof. Dr.med. Dr.rer.nat. H.-P. Gelbke

Study Director: June 17, 99
Dr.med.vet. K. Schilling

Clinical Pathology: Deckardt, June 17, 1999
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Head of Experimental Toxicology: d. 17. VI. 99
Dr.med.vet. B. Hildebrand

STATEMENT

OF THE QUALITY ASSURANCE UNIT

Number of test substance:	97/491-1
Name of test substance	Di-2-ethylhexyl Phthalate
Title:	Report: **Di-2-ethylhexyl Phthalate** - Two-Generation Reproduction Toxicity Range-Finding Study in Wistar Rats; Continuous Dietary Administration

The Quality Assurance Unit inspected the study, audited the final report and reported findings to the Study Director and to Management.

Phase of study/inspection	Date of inspection	Report to Study Director and to Management
Protocol:	Jan. 06, 1998	March 09, 1998
Conduct of study:	March 09, 1998 April 02, 1998 April 08, 1998 April 09, 1998* April 29, 1998 July 23, 1998 Aug. 11, 1998 Aug. 14, 1998	March 09, 1998 April 02, 1998 April 08, 1998 April 09, 1998 April 29, 1998 July 24, 1998 Aug. 11, 1998 Aug. 14, 1998
Audit of the study:	April 28, 1999*	April 30, 1999

*= Independently inspected by GLP-Consultancy, Grundackerstraße 18, CH-4414 Füllinsdorf.

Ludwigshafen, June 17, 1999

J. Hajok

J. Hajok
(Head of Quality Assurance Unit)

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THIS REPORT CONSISTS OF VOLUMES I, II AND III

1. SUMMARY

1.1. METHODS

Di-2-ethylhexyl Phthalate was administered to groups of 10 male and 10 female sexually immature rats (F0 parental generation) as a constant homogeneous addition to the food in different concentrations (0; 1,000; 3,000 or 9,000 ppm). Test diets containing **Di-2-ethylhexyl Phthalate** were offered continuously throughout the study. At least 70 days after the beginning of treatment, F0 animals were mated to produce the litter (F1). Mating pairs were from the same dose group and F1 animals selected for breeding were continued in the same dosing group as their parents. Groups of 9 - 10 males and 8 - 10 females were selected from F1 pups as F1 parental generation to produce a F2 litter. The terminal sacrifice of the F2 weanlings and female F1 parental animals occurred at day 2 post partum.

Food consumption of the F0 and F1 parents was determined regularly during premating (once weekly). Additionally, food consumption of the F0 females animals was measured during gestation and lactation periods as well as of F1 females animals during gestation.

Body weights of F0 and F1 parents were determined at least once weekly. The F1 pups were weighed on the day after birth and on days 4, 7, 14 and 21 post partum. The F2 pups were weighed on day 2 post partum.

The parents' and the pups' state of health was checked each day, and parental animals were examined for their mating and reproductive performances. Pups were sexed, their viability was recorded and F1 pups were monitored with respect to their sexual landmarks. Additionally, anogenital distance was measured on day 2 after birth in the F2 pups and the anogenital index was calculated, thereafter the pups were killed, the abdomen was opened to determine the internal sex and they were discarded. All F1 pups were examined macroscopically at necropsy.

Blood samples were taken from all F1 pups, pooled per sex and litter and group as well as from all female F0 parental animals and male F1 parental animals. The pooled samples from the F1 pups were analyzed within a parallel study and the results will be reported separately.

All F0 and F1 parental animals with the exception of female F1 parental animals were assessed for gross pathology. Selected organs of these animals were weighed and a histopathological examination of the testes and epididymides was performed. Additionally, one testis from one male F1 pup per litter and per group was also examined histopathologically.

The mean dose of **Di-2-ethylhexyl Phthalate** administered (during premating (F0 and F1 parental animals)) was approx. 110 mg/kg body weight/day in the 1,000 ppm group, approx. 339 mg/kg body weight/day in the 3,000 ppm group and approx. 1060 mg/kg body weight/ day in the 9,000 ppm group.

1.2. RESULTS

The following substance-related findings were obtained:

9,000 ppm (about 1,060 mg/kg body weight/day)

F0 parental animals

CLINICAL EXAMINATIONS AND REPRODUCTIVE PERFORMANCE

- statistically significantly reduced food consumption in F0 females
- statistically significantly reduced body weight/body weight gain in F0 females
- statistically significantly increased postimplantation loss (31% versus 10% in the control)

ORGAN WEIGHTS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- significantly increased mean absolute and relative liver weights in males and females

F1 pups

CLINICAL EXAMINATIONS

- reduced total number of delivered pups and statistically significantly mean number of delivered pups/dam (66%)
- slightly increased pup mortality during day 0 and 4 p.p. (reduced viability index)
- lower mean body weights in both sexes until weaning and statistically significantly retarded body weight gain
- increased incidence of males with the presence of areolas/nipple anlagen (83.9%)
- delay in vaginal opening and preputial separation in the selected F1 animals due to delay in physical development as indicated by reduced body weight

HISTOPATHOLOGICAL FINDINGS
(indicated with I1 in the tables)

- loss of spermatocytes in most animals (7/9)

F1 parental animals

CLINICAL EXAMINATIONS AND REPRODUCTIVE PERFORMANCE

- premature death of 3 out of 9 males and 2 out of 9 females in the first week of the premating phase
- initially statistically significantly reduced food consumption in both sexes during the first one to two weeks of the premating phase
- initially statistically significantly reduced mean body weights in both sexes during the premating phase

- reduced mean body weights and statistically significantly retarded body weight gain in females during gestation
- reduced fertility in male animals (fertility/mating index: 83%)

ORGAN WEIGHTS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- significantly increased mean absolute and relative liver weights in male rats
- significantly decreased mean absolute and relative testes weights
- reduced size of the testes in three of six animals
- focal or diffuse atrophy of spermatogenesis in the testes
- diffuse Leydig cell hyperplasia in all animals
- interstitial edema in the testes in three of six animals
- significantly decreased mean absolute weight of the epididymides
- reduced size of the epididymides in three of six animals
- debris of an altered spermatogenesis in the epididymides of almost all animals (5/6)
- aspermia in two animals
- the seminal vesicle not detectable/missing in one of six animals
- areola(e)/nipple(s) present in the mammary gland area of one of six males

F2 pups

CLINICAL EXAMINATIONS

- reduced total number of delivered pups and statistically significantly reduced mean number of delivered pups/dam (66%)
- statistically significantly reduced anogenital distance (87%) and index in male pups

3,000 ppm (about 339 mg/kg body weight/day)

F0 parental animals

CLINICAL EXAMINATIONS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- no substance-related adverse effects in F1 males or females

ORGAN WEIGHTS

- significantly increased mean absolute liver weight in females
- significantly increased mean relative liver weight in males and females

F1 pups

CLINICAL EXAMINATIONS

- no substance-related adverse effects

HISTOPATHOLOGICAL FINDINGS

- loss of spermatocytes in few animals (2/10)

F1 parental animals

CLINICAL EXAMINATIONS FINDINGS

- no substance-related adverse effects in F1 males or females

ORGAN WEIGHTS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- no treatment related weight changes, gross lesions or microscopic findings.

F2 pups

CLINICAL EXAMINATIONS

- no substance-related adverse effects

1,000 ppm (about 110 mg/kg body weight/day)

F0 parental animals

CLINICAL EXAMINATIONS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- no substance-related adverse effects in F0 males or females

ORGAN WEIGHTS (indicated with F1 in the tables)

- significantly increased mean absolute liver weight in females

F1 pups

CLINICAL EXAMINATIONS

- no substance-related adverse effects

HISTOPATHOLOGICAL FINDINGS

- no treatment related microscopic findings

F1 parental animals

CLINICAL EXAMINATIONS

- no substance-related adverse effects in F1 males or females

ORGAN WEIGHTS/GROSS AND HISTOPATHOLOGICAL FINDINGS

- no treatment related weight changes, gross lesions or microscopic findings.

F2 pups

CLINICAL EXAMINATIONS

- no substance-related adverse effects

1.3. CONCLUSION

Under the conditions of this range-finding study **Di-2-ethylhexyl Phthalate** had **no adverse effects on reproductive parameters** of the male and female F0 parental animals and of the female F1 parental animals of all groups (1,000; 3,000 and 9,000 ppm) neither in the clinical nor in the (limited) pathological examinations. However, some indications for **reduced fertility** were observed in the male F1 parental animals at 9,000 ppm.

Evidence of **systemic effects** in the form of increased liver weights occurred from 1,000 ppm onward for female F0 parental animals, from 3,000 ppm onward for male F0 parental animals and at 9,000 ppm for male and female F1 parental rats. At 9,000 ppm toxicity was characterized by premature deaths of male and female F1 parental animals, reduced food consumption in F0 females and initially in F1 parental animals during premating, impaired body weight/body weight gain in female F0 and F1 parental animals as well as initially in male F1 parental rats during premating.

Substance-induced signs of **developmental toxicity** were noted at 9,000 ppm for the progeny of the F0 and F1 parents in the form of reduced total number and statistically significantly reduced mean number of delivered F1 and F2 pups with a concurrent increase in postimplantation loss for the F0 females, increased pup mortality in the F1 pups and impaired weight gain in F1 pups. Furthermore, at 9,000 ppm a statistically significant reduction in the anogenital distance/index was recorded in F2 male pups, while in F1 pups there was an increased incidence of males with the presence of areolas/nipple anlagen. Additionally, a delay in vaginal opening and preputial separation in the selected F1 animals was noted as sign of a general delay in physical development. Moreover, a dose-dependent increase in loss of spermatocytes was noted in the male F1 pups at 3,000 and 9,000 ppm. However, 1,000 ppm did not affect the progeny at all.

Based on these data the following no observed adverse effect levels (NOAEL's) were derived:

The NOAEL for reproductive function is 9,000 ppm (about 1,060 mg/kg body weight/day) for the male and female F0 parental and female F1 parental rats, while the NOAEL for the male F1 parental rats is 3,000 ppm (about 339 mg/kg body weight/day).

The NOAEL for general, systemic findings of the test substance is 1,000 ppm (about 110 mg/kg body weight/day) for the F0 and F1 males and F1 females , while for the female F0 parental animals it is below 1,000 ppm.

The NOAEL for developmental toxicity for the F1 litter could be fixed at 1,000 ppm (about 110 mg/kg body weight/day) and at 3,000 ppm (about 339 mg/kg body weight/day) for the F2 litter.

2. INTRODUCTION AND DOSE SELECTION

2.1. AIM OF THE STUDY

The objective of this study was to gain experience about the effects of **Di-2-ethylhexyl Phthalate** on the reproductive performance and function as well as on growth and development of offspring from two successive generations of rats continuously administered the test substance in the diet and to enable the selection of dose levels for a subsequent 2-generation toxicity study. This report presents data collected from 2 litters (F1 and F2) produced by two generations of rats (F0 and F1) through terminal sacrifice of F2 litter and F1 adult animals.

The study was carried out from January 05, 1998 (beginning of administration to the F0 parental animals) to August 21, 1998 (last scheduled sacrifice of the F1 parental animals).

2.2. SELECTION OF DOSES/CONCENTRATION

For this range-finding study concentrations of 0; 1,000; 3,000 and 9,000 ppm were chosen by the sponsor.

2.3. TEST GUIDELINES

The study was roughly based on the following test guidelines:

- EC Commission Directive 87/302/EEC of November 18, 1987; Part B: Methods for the determination of toxicity: Two-generation reproduction toxicity test; Official Journal of the European Communities; No. L 133, pp. 47 - 50 (1988)
- OECD Guidelines for Testing of Chemicals No. 416 (Draft April 1996)
- EPA, Health Effects Test Guidelines; OPPTS 870.3800: Reproduction and Fertility Effects (August 1998)

3. MATERIAL AND METHODS

3.1. TEST SUBSTANCE

Name of test substance:	Di-2-ethylhexyl Phthalate
Lab internal abbreviation	DEHP
Test substance No.:	97/491-1
CAS No.:	117-81-7
Batch No.:	22516
Date of production:	November 13, 1997
Degree of purity:	99,7 % (method: gas chromatography; analytical report dated December 01, 1997)
Stability:	Proven by analysis (analytical report 98L00598)
Homogeneity:	Proven analytically (method: gas chromatography; analytical report dated December 01, 1997)
Physical state/appearance:	Liquid/clear/colorless
Storage conditions:	Room temperature in non-plastic (i.e. stainless unlacquered steel) containers
Characterization:	Further details on the characterization of the test substance are included in the raw data.
Analytical laboratory:	Central Analytical Department of BASF Aktiengesellschaft
Safety precautions:	The usual precautions for handling of chemicals must be observed.

Detailed description of the extent of the analytical investigations and of the analytical methods employed are stored with BASF Aktiengesellschaft.

3.2. TEST ANIMALS

3.2.1. Species and strain

Male and female Wistar rats (Chbb = THOM (SPF)) supplied by Karl THOMAE GmbH, Biberach/Riss, FRG, which were free from any clinical signs of disease, were used for the investigations. The females were nulliparous and non-pregnant at the beginning of the study. According to a written statement from the breeder, male and female animals were derived from different litters. This was necessary to rule out the possibility of sibling mating. The animals were received on December 22, 1997.

These animals were taken to form the F0 generation parental animals. All other animals used in this study (F1 and F2 generation pups and the F1 generation parental rats [raised F1 pups]) were derived from these animals.

3.2.2. Animal identification

The rats of the parental generation (F0 and F1 generations) were identified uniquely by ear tattoo. The unit digit of the animal number was tattooed on the outside of a rat's left ear, the ten digit on the inside of the left ear.

All live pups were identified by skin tattoo on day 1 post partum (p.p.) and with picric acid between days 10 and 15 after birth.

3.2.3. Reason for species selection

This strain was selected since extensive historical control data was available on Wistar rats and the rat is the preferred animal species for reproduction studies according to the different test guidelines.

3.3. HOUSING AND DIET

During the study period, the rats were housed individually in type DK III stainless steel wire mesh cages supplied by BECKER & CO., Castrop-Rauxel, FRG (floor area of about 800 cm^2), with the following exceptions: from day 18 of gestation until day 14 after birth, the pregnant animals and their litters were housed in Makrolon type M III cages. The M III cages were again supplied by BECKER & CO.. Pregnant females were provided with nesting material (cellulose wadding) toward the end of gestation.

The cages with the test animals were arranged on the racks in such a way that uniform experimental conditions (ventilation and light) were ensured.

The animals were accommodated in fully air- conditioned rooms (floor area about 22 m^2) in which central air conditioning guaranteed a range of temperature of 20 - 24°C and a range of relative humidity of 30 - 70%. There were no or only minimal deviations from these limits.

The day/night rhythm was 12 hours (12 hours light from 6.00 a.m. to 6.00 p.m. and 12 hours darkness from 6.00 p.m. to 6.00 a.m.) in general.

Before use each room was completely disinfected using a disinfector ("AUTEX" fully automatic, formalin-ammonia-based terminal disinfection). Usually, each week the walls and the floor were cleaned with water containing about 0.5% Mikro-Quat (supplied by ECOSAN GmbH, FRG).

The food used was ground Kliba laboratory diet rat/mouse/hamster maintenance, supplied by KLINGENTALMÜHLE AG, Kaiseraugst, Switzerland, which was available to the animals ad libitum throughout the study (from the day of supply to the day of or the day before necropsy). Drinking water was supplied from water bottles (ad libitum).

The bedding used throughout the study was Ssniff (type 3/4) supplied by SSNIFF SPEZIALDIÄTEN GmbH, Soest, FRG.

3.4. TEST GROUPS, CONCENTRATIONS AND NUMBERS OF THE TATTOOED ANIMALS

3.4.1. F0 generation parental animals

Test group	Concentration (ppm)	Number of animals		Animal number	
		Male	Female	Male	Female
0	0	10	10	1 - 10	41 – 50
1	1,000	10	10	11 - 20	51 – 60
2	3,000	10	10	21 - 30	61 – 70
3	9,000	10	10	31 – 40	71 – 80

3.4.2. F1 generation parental animals

Test group	Concentration (ppm)	Number of animals		Animal number	
		Male	Female	Male	Female
10	0	10	10	41-1, 42-1, 43-1, 44-1, 45-1, 46-1, 47-1, 48-1, 49-1, 50-1	41-6, 42-9, 43-10, 44-9, 45-12, 46-8, 47-8, 48-5, 49-10, 50-8
11	1,000	10	10	51-1, 52-1, 53-1, 54-1, 55-1, 56-2, 57-1, 58-1, 59-1, 60-1	51-9, 52-9, 53-6, 54-8, 55-13, 56-8, 57-2, 58-10, 59-10, 60-8
12	3,000	9	8	61-1, 62-6, 63-1, 65-3, 66-1, 67-1, 68-1, 69-3, 70-1	61-7, 62-8, 63-10, 65-10, 66-6, 67-13, 68-8, 70-7
13	9,000	9	9	71-2, 72-1, 73-1, 74-2, 75-2, 76-1, 77-3, 79-1, 80-1	71-6, 72-3, 73-8, 74-8, 75-7, 76-4, 77-7, 79-5, 80-5

3.5. TEST SUBSTANCE PREPARATION AND ANALYSES

3.5.1. Preparations of the mixtures of food and test substance

The test substance preparations were weighed in a beaker (depending on the dose group) and thoroughly mixed with a small amount of food using a spatula. Subsequently a premix was then adjusted in a Hobart mixer. This premix was then adjusted to the concentration desired with the appropriate amounts of food and mixed in a laboratory mixer of Gebr. Lödige for about 10 minutes. Instead of the laboratory mixer of Gebr. Lödige the test substance preparations were mixed in a Ruberg laboratory mixer (EM 100) since May 07, 1998. The preparation frequency was weekly with the exception that towards the end of the study the interval was up to 4 weeks. The test substance preparations were stored in non-plastic (i.e. stainless unlacquered steel) containers.

3.5.2. Analyses

All analyses mentioned under 3.5.3. were carried out at the Analytical Laboratory of the Department ZHT of BASF Aktien-gesellschaft, FRG.

3.5.3. Analyses of the test substance preparations

Analytical verifications of the stability of the test substance in the diet for a period of 32 days were carried out before the study was initiated.

Homogeneity analyses of the test substance preparations in the diet were determined in the lowest and highest concentration before the beginning of this study.

Concentration control analysis were carried out at the beginning of the study and thereafter at intervals of about three months.

To verify the accuracy of the new method of making the test substance preparations an additional verification of the concentration and the homogeneity of the lowest and highest concentrations were carried out. After the verification of the homogeneity the new mixtures were applied.

3.5.4. Analytical methods

The test substance was analyzed by gas chromatography.

More details on the methods used for the analytical investigations of the test substance preparations can be found in Volume III (Supplement: 1. Analyses of the suspensions of the test substance).

3.5.5. Food analyses

The food used in the study was assayed for chemical as well as for microbiological contaminants.

3.5.6. Drinking water analyses

The drinking water is regularly assayed for chemical contaminants by the municipal authorities of Frankenthal and the Technical Services of BASF Aktiengesellschaft as well as for the presence of microorganisms by a contract laboratory.

3.5.7. Bedding analyses

The bedding is regularly assayed for contaminants (chlorinated hydrocarbons and heavy metals).

3.6. EXPERIMENTAL PROCEDURE AND TIME SCHEDULE

3.6.1. F0 generation parental animals and their progeny

The 92 male and 132 female rats were 28 (± 1) days old when they arrived from the breeding facilities. During an acclimatization period of about 14 days, animals with lowest and highest body weights were removed from the study. The 40 male and 40 female animals required for the study were 42 (± 1) days old at the beginning of treatment, and their mean weights and weight ranges were:

- male animals: 201.0 (180.6 – 222.3) g
- female animals: 145.5 (129.7 – 161.9) g

The assignment of the animals to the different test groups was carried out using a randomization program (NIJENHUIS, A. and WILF, H.S.; 1978), according to their weight six days before the beginning of the administration period (day -6).

After the acclimatization period, the F0 generation parental animals continuously received the test substance at the appropriate concentrations in the diet until or up to about 16 hours before they were sacrificed.

At least 70 days after the beginning of treatment, males and females from the same dose group were mated at a ratio of 1 : 1.

The females were allowed to litter and rear their pups **(F1 generation pups)** until day 21 p.p. All male and all female F1 generation pups with the exception of one male and one female pup/litter (each first surviving pup/sex) were sacrificed on day 21 p.p. After decapitation of these pups blood samples were taken but were analyzed within a parallel study. The liver of all male and female pups were taken and cut in two pieces. The samples were pooled per litter and sex and was stored deep frozen to be available for possible future examinations. Also the right testis was taken, pooled per litter and stored deep frozen to be available for possible future examinations, while the left testis was pooled per litter, fixed in BOUIN's solution and one of these testes per litter was examined histopathologically (see 3.9.3.). After weaning the parental animals were killed and after decapitation blood samples were taken from the female F0 parental animals and were stored deep frozen to be available for possible future examinations. After liver weight determination the liver of the each F0 female was cut into two pieces and was separately stored deep frozen to be available for possible future examinations.

3.6.2. F1 generation parental animals and their progeny

The selected pups **(F1 generation pups)** were reared for at least 10 weeks to become the F1 generation parental animals.

The male and female F1 generation parental animals were mated with each other and the partners were assigned to one another by lot. Mating among siblings was avoided. After the mating period the **male animals** of the F1 generation parental animals were killed and after decapitation blood samples were collected and stored deep frozen to be available for possible future examinations and selected organs were weighed, preserved and histopathologically examined (see 3.9.3.) The female animals of the F1 generation were allowed to deliver and to rear the pups (**= F2 generation pups**) until day 2 p.p. (post partum). Thereafter, the female F1 generation parental animals were sacrificed and discarded.

The anogenital distance was measured (for details see 3.7.2.2.) in all **live F2 generation pups** on day 2 post partum. Thereafter, these pups were sacrificed and the internal sex was determined. Then they were discarded.

3.6.3. Pups after weaning

With the exception of the F1 generation pups, which were chosen as the basis of the F1 generation parental animals, all pups were sacrificed (by means of CO_2) after weaning.

These pups, including stillborn pups and those that died during their rearing period, were subjected to a macroscopic (external and visceral) examination. Thereafter these pups were treated as described later (see 3.7.2.6.).

3.6.4. Matings of F0 and F1 generation parental animals

Each of the male and female animals were mated overnight at a 1 : 1 ratio for a maximum of 2 weeks with the exception of male No. 68-1 which had no mating partner and male No. 71-2 at a 1 : 2 ratio. Throughout the mating period, each female animal was mated with a predetermined male animal from the same dose group.

Normally, the female animals were placed toward 4.00 p.m. in the cage with a male partner and were again separated from the male animal between 7.00 and 9.00 a.m. of the following morning. Deviations from the specified times were possible on weekends and public holidays and were reported in the raw data.

A vaginal smear was prepared after each mating and examined for sperms. If sperms were detected, pairing of the animals was discontinued. The day on which sperms were detected was denoted "day 0" and the following day "day 1 of gestation (i.e., day 1 p.c. (post coitum)).

3.6.5. Time schedule and study sequences

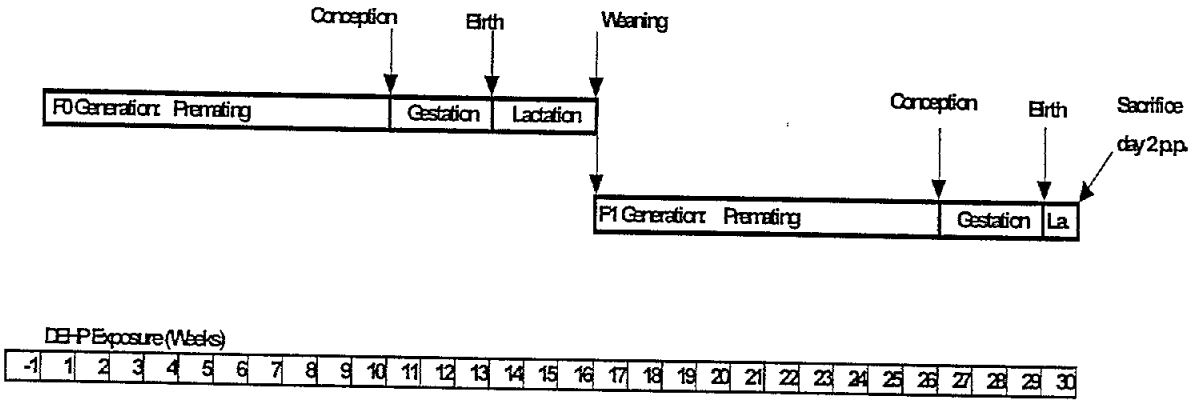
In the following schedule the relevant intervals for certain study phases/examinations are given.

Fig. 3.6.7.1.: Time schedule

Phase of study/examination	F0 generation parental animals and progeny	F1 generation parental animals and progeny
Arrival of the animals	Dec. 22, 1997	not relevant
Acclimatization period	Dec. 22, 1997 - Jan. 04, 1998	not relevant
Administration period	Jan. 05 – May 03, 1998	May 06 – Aug. 21, 1998
Mating period* for litter	F1: March 16 – March 20, 1998	F2: July 19 – Aug. 02, 1998
Gestation period for litter	F1: March 17 – April 11, 1998	F2: July 20 – Aug. 18, 1998
Birth* of litter	F1: April 07 – April 12, 1998	F2: Aug. 10 – Aug. 19, 1998
Lactation period* for litter	F1: April 07 – May 03, 1998	F2: Aug. 10 – Aug. 21, 1998
Sacrifice of litter (after weaning)	F1: April 28 – May 03, 1998	F2: Aug. 12 – Aug. 21, 1998
Sacrifice of parental animals**	♂: April 29, and April 30, 1998 ♀: April 29 – May 03, 1998	♂: Aug 11, 1998 ♀: Aug 12 - Aug 21, 1998

The study sequences can be seen from the following figure:

Fig. 3.6.7.2.: Study sequences



* Dates given are not precise, but give only the actual study phase

** Before necropsy food was withdrawn for about 16 hours

3.7. CLINICAL EXAMINATIONS AND EXAMINATION OF REPRODUCTIVE PERFORMANCE

3.7.1. Parental animals

3.7.1.1. Mortality

At least once daily a check was made for dead or moribund animals. The examinations of these animals were carried out according to the methods described under 3.9. PATHOLOGY.

3.7.1.2. Clinical observations

All parental animals were checked daily for clinically evident signs of toxicity. For technical reasons, however, the clinical observations recorded during the premating periods were printed out on a weekly basis (the daily observations can be found in the raw data).

The **nesting, littering** and **lactation behavior** of the dams was generally evaluated in the mornings in connection with the daily clinical inspection of the dams. Only special findings (e.g., animal could not litter, umbilical cord not cut) were documented on an individual dam basis.

The **littering behavior** of the dams was also inspected on weekdays (except holidays) in the mornings and in the afternoons.

The day of littering was considered to be the 24-hour period from about 3.00 p.m. of one day until about 3.00 p.m. of the following day.

3.7.1.3. Food consumption

During the premating period food consumption was determined once a week (each time for a period of 7 days) for males and females.

After the 10th (F0 generation parental animals) or 14th (F1 generation parental animals) test week, food consumption of the **females during pregnancy** (animals with evidence of sperm) was determined for days 0 - 7 , 7 - 14 and 14 - 20 p.c..

During the **lactation period** (animals with litter) food consumption was determined on days 1 - 4 , 4 - 7 and 7 - 14 p.p. with the exception of the F1 generation parental animals.

Food consumption was not determined between days 14 and 21 after parturition as required in the test guidelines cited under 2.3., since during this time pups will begin to consume considerable amounts of solid food offered, and therefore these data would be misleading.

Food consumption of the males was not determined any longer after the 10th (F0 generation parental animals) or 14th (F1 generation parental animals) test week through sacrifice. Furthermore, there was no determination of food consumption in the **males** during the mating periods, in the **females without positive evidence of sperm** during the programmed **gestation phase**, or in the **females without litters** during the **lactation phase**.

3.7.1.4. Body weight data

In general, the body weight of the **male and female parental animals** was determined once a week at the same time of the day (in the morning); if possible, the weighings was carried out until the end of the study.

The body weight change of the animals was calculated from these results.

The following exceptions are notable for the **female animals:**

a) During each mating period the F0 and the F1 generation parental females were weighed on the day of positive evidence of sperm (day 0 p.c.) and on days 7, 14 and 20 post coitum.

b) Females showing **no positive evidence of sperm** in vaginal smears were **not** weighed during the mating interval.

c) F0 females **with litter** were weighed on the day after parturition (day 1 p.p.) and on days 4, 7, 14 and 21 post partum.

d) F1 females **with litter** were weighed on day 2 p.p.

e) Females **without litter** were **not** weighed during the lactation phase.

3.7.1.5. Intake of test substance

The intake of test substance was calculated from the amount of food consumed and expressed in mg/kg body weight per day.

The calculation of the group values/day was carried out according to the following formula:

$$IT_x = \frac{FC_x \times D}{BW_y}$$

IT_x = intake of test substance on day x in mg/kg body weight/day

FC_x = daily food consumption on day x in grams

D = dose in ppm

BW_y = body weight on day y in grams (last weighing before day x)

The values listed in the Summary Tables are group means determined from the daily intakes of test substance by the individual animals.

3.7.1.6. Male reproduction data

The mating partners, the number of mating days until vaginal sperm could be detected in the female, and the gestational status of the female were noted for F0 and F1 breeding pairs.

For the **males**, mating and fertility indices were calculated for F1 and F2 litters according to the following formulas:

$$\textbf{Male mating index (\%)} = \frac{\text{number of males with confirmed mating*}}{\text{number of males placed with females}} \times 100$$

* defined by a female with vaginal sperm or that gave birth to a litter or with fetuses in utero

$$\textbf{Male fertility index (\%)} = \frac{\text{number of males proving their fertility*}}{\text{number of males placed with females}} \times 100$$

* defined by a female giving birth to a litter or with pups/fetuses in utero

3.7.1.7. Female reproduction and delivery data

The mating partners, the number of mating days until vaginal sperm could be detected, and gestational status were recorded for F0 and F1 females.

For the females, mating, fertility and gestation indices were calculated for F1 and F2 litters according to the following formulas:

$$\text{Female mating index (\%)} = \frac{\text{number of females mated*}}{\text{number of females placed with males}} \times 100$$

* defined as the number of females with vaginal sperm or that gave birth to a litter or with fetuses in utero

$$\text{Female fertility index (\%)} = \frac{\text{number of females pregnant*}}{\text{number of females mated**}} \times 100$$

* defined as the number of females that gave birth to a litter or with pups/fetuses in utero

** defined as the number of females with vaginal sperm or that gave birth to a litter or with fetuses in utero

$$\text{Gestation index (\%)} = \frac{\text{number of females with live pups on the day of birth}}{\text{number of females pregnant*}} \times 100$$

* defined as the number of females that gave birth to a litter or with fetuses in utero

Furthermore, the total number of pups delivered and the number of liveborn and stillborn pups were noted, and the live birth index was calculated for F1 and F2 litters according to the following formula:

$$\text{Live birth index (\%)} = \frac{\text{number of liveborn pups at birth}}{\text{total number of pups born}} \times 100$$

3.7.2. Litters/pups

3.7.2.1. Litter data

3.7.2.1.1. Pup number and status at delivery

All pups derived from the F0 parents (F1 litters) and the F1 parents (F2 litter) were examined as soon as possible on the day of birth to determine the total number of pups and the number of liveborn and stillborn members of each litter. Pups which died before the first determination of their status on the day of birth were designated as stillborn pups.

3.7.2.1.2. Pup viability/mortality

In general, a check was made for any dead or moribund pups twice daily on workdays (once in the morning and once in the afternoon) or as a rule, only in the morning on Saturdays, Sundays or public holidays. Dead pups were evaluated by the methods which will be described in detail in section 3.7.2.6.

The number and percentage of dead pups on the day of birth (day 0) and of pups dying between days 1-4, 5-7, 8-14 and 15-21 of the lactation period were determined; however, pups which died accidentally, had to be sacrificed due to maternal death or if applicable F2 pups sacrificed on day 2 p.p. were not included in these calculations. The number of live pups/litter was calculated on the day of birth, and on lactation days 4, 7, 14 and 21. Furthermore, viability and lactation indices were calculated according to the following formulas:

$$\text{Viability index (\%)} = \frac{\text{number of live pups on day 4 after birth}}{\text{number of live pups on the day of birth}} \times 100$$

$$\text{Lactation index (\%)} = \frac{\text{number of live pups on day 21 after birth}}{\text{number of live pups on day 4 after birth}} \times 100$$

3.7.2.1.3. Sex ratio

On the day of birth (day 0) the sex of the pups was determined by observing the distance between the anus and the base of the genital tubercle; normally, the anogenital distance is considerably greater in male than in female pups. Subsequently the sex of the pups was assessed by the external appearance of the anogenital region and/or the mammary line of the animals and was finally confirmed at necropsy.

The sex ratio was calculated for F1 and F2 pups at day 0 and for F1 pups at day 21 after birth according to the following formula:

$$\text{Sex ratio} = \frac{\text{number of live male or female pups on day 0/21}}{\text{number of live male and female pups on day 0/21}} \times 100$$

3.7.2.2. Anogenital distance measurements in F2 pups

Anogenital distance (defined as the distance from the anus [center of the anal opening] to the base of the genital tubercle) measurements were done in a blind randomized fashion, using a measuring ocular on all live male, female and uncertain F2 pups on the day 2 after birth.

3.7.2.3. Anogenital index calculations in F2 pups

The anogenital index was calculated according to the following formula:

$$\text{anogenital index} = \frac{\text{anogenital distance [mm]}}{\text{pup weight [g]}}$$

3.7.2.4. Pup body weight data

The F1 pups were weighed on the day after birth (day 1 p.p.) and on days 4, 7, 14 and 21 after birth, while the F2 pups were only weighed on day 2 p.p.

Pups' body weight change was calculated from these results with the exception of F2 pups where no body weight change could be determined.

The individual weights were always determined at about the same time of the day (in the morning).

In the relevant summary tables pup body weights and pup body weight gains are listed for males, females and males + females.

3.7.2.5. Pup clinical observations

All live pups were examined each day for clinical symptoms (including gross-morphological findings, e.g., presence of areolas/nipples).

3.7.2.6. Pup necropsy observations

After decapitation of the pups (both sexes) on day 21 p.p., blood samples (pooled blood of all male pups/litter and pooled blood of all female pups/litter) were taken but were and analyzed in a parallel study.

The pups were examined externally, eviscerated and their organs were assessed macroscopically.

Liver of all F1 pups was cut into two pieces. The samples were pooled per litter and sex and stored deep frozen (approx. - 80 °C) in order to be available for possible future examinations.

The right testis of all F1 pups were taken, pooled per litter and stored deep frozen (approx. - 80 °C) in order to be available for possible future examinations.

The left testis of all F1 pups were pooled per litter, fixed in BOUIN's solution and one of these testes per litter was examined histopathologically (see 3.9.3.).

After their macroscopic evaluation all pups were discarded.

3.7.2.7. Sexual landmarks (signs of sexual maturation)

The signs of sexual maturation the selected male and female F1 pups were monitored on the following days:

- from day 25 post partum onwards: opening of vagina
- from day 40 post partum onwards: preputial separation

The examinations were carried out in the morning. After the occurrence of preputial separation all male animals were additionally examined for external abnormalities of the genital organs using a magnification lamp.

3.7.3. Statistics of the clinical examinations*

Statistical analyses were performed according to following tables:

Parameter	Statistical test	Markers in the tables	References
Food consumption (parental animals), body weight and body weight change (parental animals and pups; for the pup weights the litter means were used), number of mating days, duration of gestation, number of pups delivered per litter	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means	* for $p \leq 0.05$ ** for $p \leq 0.01$	DUNNETT, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. JASA, Vol. 50, 1096 – 1121 DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. Biometrics, Vol. 20, 482 - 491

* Note: For the parameters food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during the different time intervals (premating, gestation and/or lactation); they are not exactly precise values, because the size of the intervals taken for calculation may differ (especially during gestation and lactation periods). For the "mean of means" values no statistical analysis was performed.

Parameter	Statistical test	Markers in the tables	References
Male and female mating index, male and female fertility index, gestation index, females with liveborn pups, females with stillborn pups, females with all stillborn pups, live birth index, pups stillborn, pups died, pups cannibalized, pups sacrificed moribund, viability index, lactation index, number of litters with affected pups at necropsy	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions	* for $p \leq 0.05$ ** for $p \leq 0.01$	Siegel S. (1956): Non-parametric statistics for behavioural sciences. McGraw-Hill New York
Proportions of affected pups per litter with necropsy observations, proportions of pups reaching special criteria in each litter concerning sexual landmarks	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	Nijenhuis, A.; Wilf H.S. (1978): Combinatorial Algorithms. Academic Press New York, 32-33 Hettmannsperger, T.P. (1984); Statistical Inference based on Ranks. John Wiley & Sons New York, 132-142

3.8. CLINICAL PATHOLOGY

Blood samples taken from F1 pups, pooled for all male pups/litter and all female pups/litter were analyzed but will be reported in a parallel study.

All other blood samples taken from female F0 and male F1 parental animals will be stored deep frozen (approx. – 80 °C) in order to be available for possible future examinations.

3.9. PATHOLOGY

3.9.1. Necropsy

The F0 generation parental animals and F1 male parental animals were sacrificed by decapitation under CO_2 anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology.

3.9.2. Organ weights

The following weight parameters of all animals of the F0 generation parental animals (F0) or the F1 generation parental animals (F1) sacrificed at scheduled dates were determined:

1. anesthetized animals (F0, F1)
2. liver (F0, F1 males)
3. testes (F0, F1)
4. ovaries (F0)
5. epididymides (F1)
6. prostate gland (F1)
7. seminal vesicle (F1)
8. coagulating gland (F1)
9. kidneys (F1 males)

3.9.3. Histopathology

Of the F0 generation parental animals, the following organs or tissues were fixed in 4% formaldehyde (F) or BOUIN's (B) solution:

1. all gross lesions (F)
2. vagina (F)
3. uterus (F)
4. testes (B)
5. epididymides (B)
6. seminal vesicle (F)
7. coagulating glands (F)
8. prostate gland (F)
9. pituitary gland (F)
10. kidneys (F)

Of the male F1 pups, of those that were not reared to produce the male F1 generation parental animals, one testicle was fixed in BOUIN's solution.

Of the F1 generation parental animals, the following organs or tissues were fixed in 4% formaldehyde (F) or BOUIN's (B) solution:

1. all gross lesions (F)
2. testes (B)
3. epididymides (B)
4. prostate gland (F)
5. seminal vesicle (F)
6. coagulating glands (F)
7. liver (F)
8. kidneys (F)

Whenever available, both testes (F0 and F1 male parental animals and F1 male pups) and both epididymides (F0 and F1 male parental animals) were processed to Hematoxylin and Eosin stained slides and assessed by histopathology.

The male parental animals of the F0 generation are to be identified by their animal numbers as outlined in the study protocol.

In the pathology tables, the male F1 pups are identified by the number of their mother as outlined in the study protocol, followed by a dash (" - ") and a "30" (e. g. 41 - 30 represents the first male pup of the control group and 80 - 30 represents the last male pup of the high dose group).
As this procedure was accepted only after the slides for these animals had already been prepared, the blocks and slides of these animals are identified by the animal number of the mothers as outlined in the study protocol, followed by "BZ" (e. g. 41 BZ represents the first male pup of the control group and 80 BZ represents the last male pup of the high dose group).

The male parental animals of the F1 generation are to be identified by the number of their mother as outlined in the study protocol, followed by a dash (" - ") and a one digit number (e. g.1, 2, 3, or 6: by this, 41 - 1 represents the first male of control group and 80 -1 represents the last male of the high dose group).

When assessing the testes histopathologically, background physiologic conditions were separated from acquired pathologic findings in order to avoid diagnostic confusion, especially when they refer to the same functional unit (seminiferous tubules) or target cells (cells of spermatogenesis).
As focal or diffuse tubular atrophy are possible treatment related findings, they were separated from "tubuli aberrantes" which are visible at the transition between testis and epididymis, and they appear as small subcapsular "atrophic" tubuli. These tubuli do not contain cells of spermatogenesis, however, they are covered by slender cells with a basal, elongated nucleus, most likely representing Sertoli cells. The number of these "aberrant tubuli" varies between one and five (grade 1), sometimes exceeding to more than five but less than ten (grade 2) tubuli so affected. As the "aberrant tubuli" only occur at the transition site, they may be missed in slides not touching this area. Therefore, "tubuli aberrantes" are often detected only unilaterally.
As "tubuli aberrantes" represent a physiologic condition, they are not combined with those atrophic tubuli lying elsewhere in the testis.

An attempt was made to correlate the gross lesions in testes or epididymides with a meaningful histologic finding.

3.9.4. Statistics of pathology

Means and standard deviations of each test group were calculated for the variables of terminal body weight and of absolute and relative organ weights (related to terminal body weight) of the animals in each test group. Further statistical analyses were performed according to following table:

Parameters	**Statistical test**	**Markers in the tables**	**References**
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using the WILCOXON test for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	HETTMANNSPERGER, T. P. (1984): Statistical Inference based on Ranks, John Wiley & Sons New York, 132-140. International Mathematical and Statistical Libraries, Inc., 2500 Park West Tower One, Houston, Texas 77042-3020, USA, nakl-1 - nakl-3. MILLER, R. G. (1981): Simultaneous Statistical Inference Springer-Verlag New York Inc., 165-167. NIJENHUIS, A. and S. W. WILF (1978): Combinatorial Algorithms, Academic Press, New York, 32-33.